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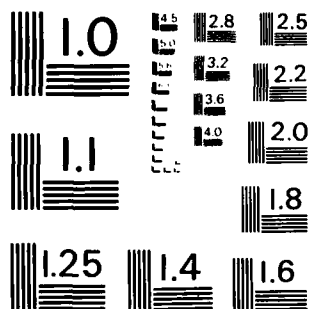
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REPORT No. 1

**PREPARATION OF HEMOGLOBIN-  
CONTAINING MICROCAPSLES**

ANNUAL REPORT

ZOILA REYES

JUNE 1981

Supported by  
U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND  
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good storage stability in a refrigerator, but because of the complexity of the encapsulation procedure, Hb was denatured when scale-up of the procedure was attempted.

The microcapsules made from lecithin-gelatin-chondroitin sulfate and from lecithin-gelatin-carrageenan were very flexible, but too large ( $>10 \mu\text{m}$ ) and had irregular shapes.

The HESA microcapsules were small, but too fragile. Those made from EC-gum arabic had good stability, ranged in size from 0.8 to 1.2  $\mu\text{m}$ , had wall thickness of 0.02 to 0.03  $\mu\text{m}$ , and had oxygen-carrying properties comparable to those of SFHb.

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## INTRODUCTION

The development of a suitable blood substitute has been under investigation for many years. An ideal blood substitute should contain substitutes for both the plasma protein and the red cells. Plasma substitutes such as hydroxyethyl starch, dextran, and gelatin have been used clinically for several years, but suitable substitutes for red blood cells have not been developed.

Stroma-free hemoglobin (SFHb) can function as plasma and red cell substitutes; however, it is rapidly removed from the circulatory system and has a high oxygen affinity that prevents release of oxygen to the tissues at normal oxygen tensions. Various chemical modifications of the structure of hemoglobin (Hb) have been studied to hinder the rapid elimination of Hb and lower its oxygen affinity. Some of these modifications improved vascular retention, but the Hb either lost its oxygen-carrying properties or showed a large increase in oxygen affinity.

Microencapsulation of SFHb is being investigated with the aim of developing Hb-containing microcapsules that show improved retention time in the circulatory systems and better oxygen-transporting properties.

## BACKGROUND AND APPROACH

In recent years, microcapsules containing Hb have been prepared and evaluated as models for red blood cells. The capsules were made from various modified natural and synthetic polymers and lipids. The Hb in these artificial cells had the ability to combine reversibly with oxygen; however, when suspensions of some of these microcapsules were intravenously injected in animals, they were rapidly removed from the circulatory system. The failure of the artificial cells to survive for a longer period of time in the blood stream was attributed to the large size of the capsules, their lack of surface charge, or the rigidity and chemical nature of the capsule membrane.

Our approach was to microencapsulate Hb with biocompatible materials to produce capsule membranes having mechanical properties similar to those of the natural erythrocytes. The materials selected for evaluation include complexes of lecithin with low-molecular-weight polysaccharides or protein, such as albumin-lecithin, gum arabic-lecithin, and collagen-mucopolysaccharides, as well as blood compatible synthetic polymers such as poly(2-hydroxyethyl methacrylate). In addition, we proposed to make capsules with diameters of 1 to 2  $\mu\text{m}$ , which is theoretically small enough to be able to traverse the capillary networks easily and large enough to reduce problems of extravasation, even when the apertures shrink to 3  $\mu\text{m}$  or less during trauma.



## RESULTS AND DISCUSSION

The microencapsulation of aqueous solutions of SFHb was studied with the following encapsulating systems:

- (1) Lecithin complexes with gelatin, gelatin-gum arabic, gelatin-chondroitin sulfate, gelatin-carrageenan, and albumin-gum arabic.
- (2) Ethyl cellulose-gum arabic.
- (3) Hydroxyethyl starch acetate-gum arabic.

The SFHb used in this work was supplied by the Letterman Army Institute of Research (LAIR) as a 28% or 30% aqueous solution, in frozen state. The Hb solution samples were thawed, filtered through a Millipore filter (0.22- $\mu$ m pore size), divided into 10-ml samples, and stored in a freezer for use in the microencapsulation tests. Some of the SFHb samples could not be filtered after thawing because they contained small amounts of solid particles. When these were removed by centrifugation at 20,000 RPM for 45 min at 10°C, the solutions could be filtered through the 0.22- $\mu$ m filter.

### Lecithin Complexes

Lecithin or phosphatidylcholine is found in the cells of all living organisms (plants and animals) and has been used to model membranes, either alone or in combination with cholesterol and long-chain anions or cations.

Since lecithin is the salt of a strong acid and strong base, it has little buffering capacity at physiological pH ranges. Its isoelectric point is expected to be close to pH 7; however, the experimental values vary because of contamination. At pH values of 6.7 to 7.5, lecithin can form complexes with negatively charged colloids such as gum arabic and also with positively charged proteins and inorganic salts. Complexed lecithins, especially in combination with proteins, are more stable toward oxidation.

Two grades of lecithin were used in the microencapsulation tests: refined soybean (ICN Nutritional Biochemicals) and egg lecithin (L- $\alpha$ -phosphatidylcholine, Sigma). Both types of lecithin were crystallized twice from petroleum ether (30-60°C) and acetone and stored under acetone in an argon atmosphere in a refrigerator. Although there was a little difference between the lecithin types, good capsules were made with both grades.

### Lecithin-Gelatin

The initial procedure used for the microencapsulation of SFHb with lecithin-gelatin consisted of the following steps:

- (1) Emulsify the Hb solution in a lecithin solution in a volatile solvent such as low-boiling petroleum ether (PE), or Freon TMC (azeotrope of methylene chloride and Freon TF, Du Pont) to form a water-in-oil (W/O emulsion. In this step, the Hb forms a complex with lecithin at the water-oil interface.
- (2) Stir the emulsion at room temperature under stream of nitrogen for 10 to 15 min to evaporate most of the solvent.
- (3) Add a 5% aqueous gelatin solution at 25°C and pH 7 and stir to disperse the lecithin-coated Hb droplets in the gelatin solution and form a W/O-in-water (W/O/W) emulsion.
- (4) Emulsify the gelatin emulsion in mineral oil containing 0.5% Span 85 (sorbitan trioleate, Atlas Chemicals), a W/O emulsifier.
- (5) Stir the emulsion in an ice-water bath to gel the gelatin and form solid Hb-containing microcapsules. During this time the residual solvent of the first W/O emulsion diffuses gradually into the mineral oil phase and is evaporated.
- (6) Drop slowly a solution of tolylene diisocyanate (TDI) in PE to crosslink the gelatin. (Some of the Hb at the interface is also crosslinked).
- (7) Stir the mixture in the ice-water bath for 30 min longer and then dilute it with an equal volume of PE.
- (8) Separate the capsules, either by decanting the top liquid layer or by centrifuging at a low speed and wash them four times with low-boiling PE.
- (9) Suspend the particles in a small amount of pentane and emulsify the suspension in a 2% aqueous solution of Tween 80 (polyoxyethylene sorbitan monooleate, Atlas Chemicals).
- (10) Stir the emulsion at room temperature under a stream of nitrogen to evaporate the pentane, then dilute the emulsion with an equal volume of deionized water and allow the capsules to settle in an ice-water bath.

- (11) Separate the capsules by decanting the top layer (if the capsules have not settled within 30 min, separate the capsules by centrifuging at a low speed), wash them five times with 0.9% aqueous NaCl solution, and suspend them in the same saline solution for storage in a refrigerator.

The ratio of Hb to capsule wall material in these microcapsules was 70:30, and the capsules had diameters of 5 to 10  $\mu\text{m}$ . The visible absorption spectrum of an oxygenated sample of the capsule suspension was similar to that of an oxygenated sample of the original Hb solution diluted with saline. However, because of the opacity of the capsule suspension, the spectrum bands were not as sharp as those of the Hb solution. Later, when we used a frosted cell in the reference beam of the spectrophotometer to correct for the opacity of the capsule suspension, we obtained the spectrum of the encapsulated Hb with more intense, well-defined bands.

In a subsequent microencapsulation test, we used a modified procedure for comparison. The Hb and gelatin solutions were combined, and the mixture was emulsified in mineral oil containing the lecithin and the Span 85. Identical amounts of materials were used. This method eliminated three steps of the previous procedure. The W/O emulsion obtained was cooled in an ice-water bath, and the gelled Hb particles were treated with a solution of TDI in PE to crosslink the gelatin. All the other steps of the procedure were the same as those of the previous one. The microcapsules obtained had a visible absorption spectrum almost identical to that of the microcapsules made by first procedure. Specific details of the procedure and amounts of materials used are given below.

These initial microencapsulation tests were conducted with 2-g samples of a 30% SFHb solution. Since this second procedure was simpler, we used it for preparing larger samples of microencapsulated Hb, using 5, 10, and 15 g of the Hb solution. However, we had problems in the scale up experiments, particularly in the emulsification in the aqueous Tween 80 solution and in the evaporation of the organic solvent from the emulsion produced (Steps 6 and 7 of the procedure described below). Large amounts of foam were produced during emulsification, and removal of the solvent required several hours during which it appeared that some of the Hb was denatured.

In spite of these problems, we did prepare a batch of very uniform microcapsules with 2 to 3  $\mu\text{m}$  diameters, from a 10-g Hb solution sample. These microcapsules were prepared in an argon atmosphere by the following procedure (all the solutions were purged with nitrogen).

- (1) A 10-g sample of a 30% Hb solution was mixed with 10 g of a 5% aqueous gelatin solution of pH 7 maintained at 25°C.

- (2) Mineral oil (40 g), 5 ml of 4% lecithin (Soybean, refined ICN Nutritional Biochemicals) in petroleum ether (PE) (30°-60°C), and 5 ml 4% Span 85 (Sorbitan trioleate, Atlas Chemical) in PE (30-60°C) were mixed.
- (3) The aqueous solution (1) was stirred into the oil solution, cooled in an ice bath, and sonicated in the ice bath for two periods of 30 sec each with an interval of 15 sec between them to produce a fine particle emulsion (1-2  $\mu$ m). An Ultra Tip sonicator (Wave Energy Systems) was used.
- (4) The fine particle emulsion was diluted with 10 ml PE (30°-60°C) and stirred mechanically in the ice bath while a solution of 0.05 g tolylene diisocyanate in 10 ml PE (30-60°C) was dropped in. After stirring in the ice bath for 30 to 40 min longer, the emulsion was diluted with an equal volume of PE (30-60°C) and centrifuged in an argon atmosphere.
- (5) The microcapsules were washed four times with low boiling PE to remove the mineral oil and then were dispersed in small volume (20 ml) of PE.
- (6) The dispersion was emulsified in 30 ml of a 2% Tween 80 (polyoxyethylene sorbitan monooleate, Atlas Chemicals) in water.
- (7) The emulsion obtained was stirred at room temperature under jets of nitrogen to evaporate the PE. Deionized water (30 ml) was added during the evaporation of the PE to dilute the emulsion.
- (8) The microcapsules were separated by centrifugation, were washed once with deionized water and four times with 0.9% NaCl solution, and then suspended in saline for storage in a refrigerator.

The visible absorption spectra of the microcapsules under argon and under oxygen showed that the Hb retained its ability to combine reversibly with oxygen. On storage in the refrigerator under argon, the capsules showed considerable discoloration within four days.

#### Lecithin-Gelatin-Gum Arabic

Gum arabic (GA), a carboxylated polysaccharide, can form complexes (ionic bonding) with both the Hb and gelatin. It was incorporated in small amounts within the capsules to improve the Hb's resistance to autoxidation and denaturation. For each 1.0 g of the 30% Hb solution, we used 0.1 g of a 10% aqueous GA solution at pH 7.

In a microencapsulation experiment conducted with 2.0 g of the Hb solution and 0.2 g of 10% GA solution, these two solutions were

mixed and stirred for a few minutes before the gelatin solution was added. The resulting solution mixture was then encapsulated with the same amounts of materials used for the lecithin-gelatin system. The visible absorption spectrum of the microcapsules obtained was almost identical to that of the gelatin microcapsules; however, the lecithin-gelatin-GA microcapsules showed greater resistance to discoloration during storage in a refrigerator.

A larger batch of microcapsules was then prepared for shipment to LAIR. The emulsifications were done in three batches to minimize foaming. The materials used for this batch are given below:

15.0 g 30% SFHb solution (this solution could not be filtered)  
1.5 g 10% GA aqueous solution, pH 7.  
15.0 g 5% gelatin aqueous solution, pH 7, at 25°C (deionized water, filtered through a 0.2- $\mu$ m filter, was used for preparation of the aqueous solutions.)  
  
45.0 g mineral oil  
7.5 ml 4% lecithin in PE (30°-60°C), filtered through a 0.2- $\mu$ m filter  
7.5 ml 4% Span 85 in PE (30°-60°C), filtered through a 0.2- $\mu$ m filter  
0.75 g tolylene diisocyanate, freshly distilled  
  
80.0 g 2% Tween 80, aqueous solution.

Because it took longer to prepare and filter these solutions, it was not possible to finish the encapsulation in one day. The procedure was stopped after the microcapsules had been emulsified in the aqueous Tween 80 solution and part of the organic solvent removed. The emulsion was stored in a refrigerator under argon. The next day, the removal of the residual solvent and washing of the microcapsules with saline were completed. Analysis of a sample of the microcapsules showed that the Hb had lost its ability to combine reversibly with oxygen. It appeared that the Hb had been denatured during the encapsulation.

It is well known that proteins are often denatured when they come into contact with organic solvents. However, an evaluation of the solvents used during encapsulation showed that these solvents had no significant effect on Hb under the microencapsulation conditions.

We had used a relatively high TDI concentration to achieve quick crosslinking of the gelatin. Some of the Hb molecules were possibly crosslinked and incorporated into the capsule membrane. However, when either lower TDI concentrations or shorter reaction times (i.e., 5 to 10 min instead of 30 min) were used, the Hb was not completely encapsulated and could be removed from the capsules by simply melting the gelatin. When the Hb/gelatin weight ratio was varied from 6/1 to 1/1 and 1/2, while maintaining the TDI concentration at the same level, the microcapsules were insufficiently crosslinked and swelled considerably after they were transferred to the aqueous media.

Because the foam produced during the transfer of the capsules to the aqueous medium could cause denaturation of Hb in some experiments we substituted aqueous solutions of GA or of hydroxyethyl starch for the Tween 80 solutions. Less foam was produced in these solutions, and at colloid concentrations from 5% to 10%, the emulsions were stable enough for satisfactory removal of the organic solvent.

To provide additional protection to the Hb during encapsulation, we decreased the Hb content of the capsules and applied a thin coating of a lecithin complex to the Hb droplets before enclosing them in the capsule wall material. A lecithin-gum arabic complex was used. This complex was initially formed by adding 1.0 g of 1% aqueous GA solution to a solution of 50 mg lecithin (either egg or soybean lecithin) in 10 g of a mixture of equal weights of PE (30°-60°C) and Freon TF. After stirring of the mixture at room temperature in a closed vial under argon for 20 min, 1.0 g 30% Hb solution and 0.1 ml Ringer Locke solution were added. Stirring was continued until the Hb droplets became coated by the smaller GA droplets.

Because it took a long time to obtain a uniform dispersion, we developed a second procedure. In this procedure, the Hb solution is mixed with the gum arabic and Ringer Locke solutions and the mixture is emulsified by sonication in the lecithin solution in an ice-water bath. The emulsion is diluted with 5 ml PE and is allowed to stand in the ice-water bath for 15 min, then most of the solvent and free lecithin are removed by centrifugation at a low speed.

The coated Hb particles can be transferred to an aqueous medium by gently washing with 0.1 M NaCl. They can also be suspended in an organic solvent such as PE and encapsulated directly by a coacervated colloid system as described below.

The complex that coats the Hb droplets is an ionic complex of lecithin-GA-Hb and appears to be stable at neutral pH. In subsequent tests with this system, a small amount of Span 85 (0.5 g of 2% Span 85 in PE) was incorporated in the lecithin solution to improve the formation and stabilize the coating complex.

#### Lecithin-GA-Gelatin-Chondroitin Sulfate

Chondroitin sulfate (CS), from Vega Chemicals, is a mucopolysaccharide that can form complex coacervates with proteins such as gelatin and Hb. The coacervates used in the microencapsulation tests were produced at pH values of 6.8 to 7.2 from CS-gelatin and CS-gelatin-gum arabic.

For encapsulation of the lecithin-coated Hb prepared as described above from 1.0 g of the Hb solution, we used a coacervate formed by mixing the following solutions: 10.0 g of 1% aqueous gelatin, pH 7, at 25°C; 0.5 g of 2% aqueous chondroitin sulfate, sodium salt, pH 7 at 25°C; and 2 drops of 2% calcium chloride. The coacervate was maintain

at 25°C in a water bath and stirred while a suspension of the lecithin-coated Hb in 1.5 ml pentane was added. The pentane evaporated quickly during stirring, and the Hb particles were coated by the gelatin-chondroitin sulfate coacervate droplets. After cooling to 10°C, solid microcapsules formed.

Microscopic examination showed that the capsules had clear, thick gelatin walls, irregular shapes, and ranged in size from 5 to 15  $\mu$ m. The Hb particles were encapsulated as clusters or aggregates of various sizes. The microcapsules were hardened by adding 0.25 ml of 12.5% glutaraldehyde, and after 2 hr of continuous stirring, the capsules were separated by centrifugation, washed with saline several times, and suspended in saline for storage in a refrigerator.

In a similar experiment conducted with 1.0 g of the Hb solution, the lecithin-coated Hb was encapsulated with a coacervate formed by mixing the following solutions: 5 g of 2% gelatin, pH 7, at 25°C; 1.0 g 0.5% chondroitin sulfate, pH 7; 5.0 g 2% GA, pH 7, at 25°C; and 4.0 g water. The coacervate was stirred at 25°-28°C while a pentane suspension of the coated Hb particles was added. After evaporation of the pentane, the mixture was gradually cooled with stirring to solidify the microcapsules.

As in the preceding experiment, the Hb particles were encapsulated in clusters and the microcapsules had irregular shapes and were large. The microcapsules were hardened by treatment with glutaraldehyde, and after repeated washing with saline, they were suspended in saline for storage in a refrigerator.

Although in these microencapsulation experiments, the Hb was not denatured, the microcapsules were too large and irregular and, therefore, were unsuitable for use as red blood cell substitutes.

#### Lecithin-Gelatin-Carrageenan

Carrageenan, a sulfated polysaccharide complex can form complex coacervates with lecithin and with proteins. Two grades of carrageenan were obtained for this work: Stamere Types N-200 sodium carrageenan (Meer Corporation) and Gelcarin HWG carrageenan (Marine Colloids Division, FMC Corporation). The Stamere type was too impure for use in this work. The gelcarin carrageenan forms clear solutions in water at 65°-70°C, and these solutions set to firm gel on cooling.

Complex coacervates of aqueous 1% gelatin and 0.5% carrageenan solutions were formed at pH 6.8-7. For encapsulation of the lecithin-coated Hb particles obtained from 1.0 g of Hb solution, we dispersed a pentane suspension of the Hb particles in 12 g of 1% aqueous gelatin at 25°C and pH 7, and added dropwise 2.0 g of 0.5% aqueous carrageenan solution at 35°C and pH 7. The coacervate formed rapidly, and the Hb particles were coated by the gelatin-carrageenan complex. The temperature of the mixture was raised to 35°C for 10 min to prevent gelling of the coacervate before evaporation of the residual pentane. Then, the mixture was cooled gradually to 10°C, and 0.25 ml of 12.5% glutaraldehyde

was added to harden the capsules. After stirring the mixture for 1.5 hr, the capsules were separated by centrifugation, washed four times with saline, and suspended in saline for storage in the refrigerator.

These capsules had very thick clear membranes, contained aggregates of the Hb particles, and, like those described above, were large ( $>10\text{ }\mu\text{m}$ ) and of irregular shapes. Attempts to prepare smaller and uniform capsules by using a higher stirring speed during the encapsulation resulted in damage to the lecithin-coated Hb particles.

#### Lecithin-Albumin-GA

The albumin (egg albumin) obtained from ICN Pharmaceuticals was suitable for encapsulation of pharmaceuticals, but contained impurities that made it unsuitable for the encapsulation of Hb. However, we conducted a few tests with it, hoping to develop a procedure that we could use with a pure grade of albumin.

The first experiment was conducted with the following solutions:

- (1) 1.0 g 28% SFHb solution, mixed with 0.2 g 5% GA aqueous solution (pH 7), 1.0 g 5% albumin solution in saline (pH 7), and 0.2 ml 5% glucose.
- (2) 0.4 g ethyl cellulose (EC) dissolved in 4 ml methylene chloride and 6 ml Freon TMC mixed with a solution of 0.020 g egg lecithin in cyclohexane.
- (3) 20 mg TDI in 3 ml cyclohexane.

The Hb solution was emulsified in solution (2). The fine particle emulsion obtained was cooled in an ice-water bath with stirring for 30 min. Solution (3) was then added slowly and stirring was continued for 15 min longer. The emulsion was diluted with 10 ml toluene, and the coated-Hb particles were separated by centrifugation and washed with toluene (to remove EC), toluene-cyclohexane, and cyclohexane, and then suspended in 5 ml PE. The suspension was emulsified in 20 ml of 2% Tween 80, and after the PE was evaporated by stirring the emulsion in a stream of nitrogen, the capsules were allowed to settle. The aqueous layer was decanted and the capsules were washed five times with saline and suspended in saline.

The capsules were small, 2 to 3  $\mu\text{m}$ , and fragile. During the saline washings some Hb was extracted from them, and they became aggregated after a few days storage in a refrigerator.

In the second test, solution (1) contained the same amounts of all the materials except albumin; a solution of 0.2 g albumin in 5 ml water was used. The second and third solutions were unchanged. The capsules were transferred to the aqueous medium by dispersing the PE suspension in 30 ml 2% Pegosperse 400 (polyethylene glycol, mono and dioleate, food



grade, from Glyco Chemicals) in water. After the solvent had evaporated, the dispersion was centrifuged.

Either very little of the Hb was encapsulated or the capsules were damaged during centrifugation, for the Hb was in the water layer and the albumin particles have practically none.

#### Ethyl Cellulose-Gum Arabic

In the encapsulation experiments conducted with ethyl cellulose (EC), no emulsifying or crosslinking agents were used. GA was used within the capsules to improve the stability of Hb and outside the capsules to impart a negative surface charge to them.

Small-scale tests conducted with 0.5 and 1.0 g samples of the Hb solution showed that Hb can be encapsulated in EC in the absence of emulsifying agents. Since no crosslinking agents were needed, and the solvents used had no effect, the Hb was not denatured. The microcapsules showed good stability, and the Hb in the capsules combined reversibly with oxygen.

A larger sample of microcapsules was prepared for evaluation at LAIR. This sample was prepared in four batches, using the following materials per batch:

##### Solution 1

5.0 g 28% SFHb solution  
2.5 g 2% gum arabic aqueous solution, pH 7  
2.5 g 3% glucose in water

##### Solution 2

2.0 g EC (Hercules N 100)  
98.0 g Freon TMC

##### Solution 3

160 g 10% gum arabic, pH 7.

The three solutions were cooled to 10°C before use. Solution 1 was emulsified in solution 2 by sonication in an ice bath for 45 sec. The fine particle emulsion obtained was added slowly to solution 3, which was stirred with a magnetic stirrer in an ice-water bath. The resulting dispersion was stirred in a Virtis homogenizer at a moderate speed for 10 min in an ice-water bath, and then it was transferred to a 1-liter beaker (previously weighed) using 50 ml deionized water to rinse the homogenizer container. The beaker and contents were weighed, and the dispersion was stirred rapidly with a magnetic stirrer under a stream of argon and nitrogen to evaporate the Freon TMC at room temperature. After 2 hr, approximately 60% of the solvent had evaporated. To ensure complete removal of the solvent, the batches were stirred overnight at room temperature under a blanket of nitrogen. The total weight loss ranged from 98.9 to 102 g per batch.

The microcapsule suspension was diluted with 50 ml saline and centrifuged. The liquid layer was decanted, and the microcapsules were washed five times with 0.9% NaCl (previously filtered through a 0.22- $\mu$ m filter) and dispersed in the saline solution. A few capsule aggregates that formed during the solvent evaporation were separated by centrifuging the saline suspension for 30 sec at a low speed.

From the combined microcapsule batches, a 95-g sample of the suspension, estimated to contain 16% microcapsules, and labeled S-1186017, was sent to LAIR for evaluation.

#### Hydroxyethyl Starch Acetate-Gum Arabic

Hydroxyethyl starch acetate (HESA) was prepared by acetylation of hydroxyethyl starch (Sigma Chemical Company) with acetic anhydride.\* In small-scale experiments conducted with solutions of HESA in chloroform or methylene chloride and 1.0-g samples of the SFHb solution, microcapsules containing Hb were produced. No crosslinking agents were used, but a small amount of Span 85, a water-in-oil emulsifier, was needed to stabilize the initial W/O emulsion.

A larger batch was prepared with the following materials:

##### Solution 1

15.0 g 28% SFHb solution  
7.5 g 2% GA solution, pH 7  
7.5 g 3% glucose

##### Solution 2

6.0 g HESA in 94.0 g chloroform  
1.2 g 2% Span 85 in chloroform

##### Solution 3

160 g 10% GA solution, pH 7.

The procedure was the same as that used for EC.

The microcapsules produced were very small, 1-2  $\mu$ m, but they were easily damaged during centrifugation and washing with saline. This was due to the brittleness of the HESA membranes, which could not withstand the pressure during centrifugation. Thus, the Hb was gradually extracted from some of the capsules during the washing operation.

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\*R. L. Lohman, Jr., J. W. Sloan, and C. E. Rist, J. Am. Chem. Soc., 72, 5717 (1950).

#### Characterization of Microencapsulated SFHb, Sample S-11860-17

The size, size distribution, and shape of the microcapsules were determined by microscopic examination at 1,260X magnification. The microcapsules were spherical and ranged from 0.6  $\mu\text{m}$  to 2.8  $\mu\text{m}$  in diameter, the major fraction had 0.8  $\mu\text{m}$  diameter. One hundred microcapsules were sized, the results are shown in Figure 1.

The microcapsule wall thickness was determined from electron photographs of cross sections of the capsules imbeded in an epoxy resin. The procedure was to centrifuge a sample of the capsules, decant the liquid, fix the microcapsules for 2 hr in 2% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2), then wash three times with 0.1 M cacodylate, and post fix them in 1%  $\text{OsO}_4$  in 0.1 M cacodylate. The microcapsules were then dehydrated in a graded series of ethanols (aqueous 50%, 70%, 95%) and three times in 100% ethanol. Finally, they were washed three times with propylene oxide, infiltrated overnight with a 50:50 propylene oxide:araldite (epoxy resin), and imbeded in the resin. Sections, 100 nm, cut for electron microscopy were stained with uranyl acetate followed by lead citrate. The microcapsule wall thickness ranged from 0.02 to 0.03  $\mu\text{m}$ .

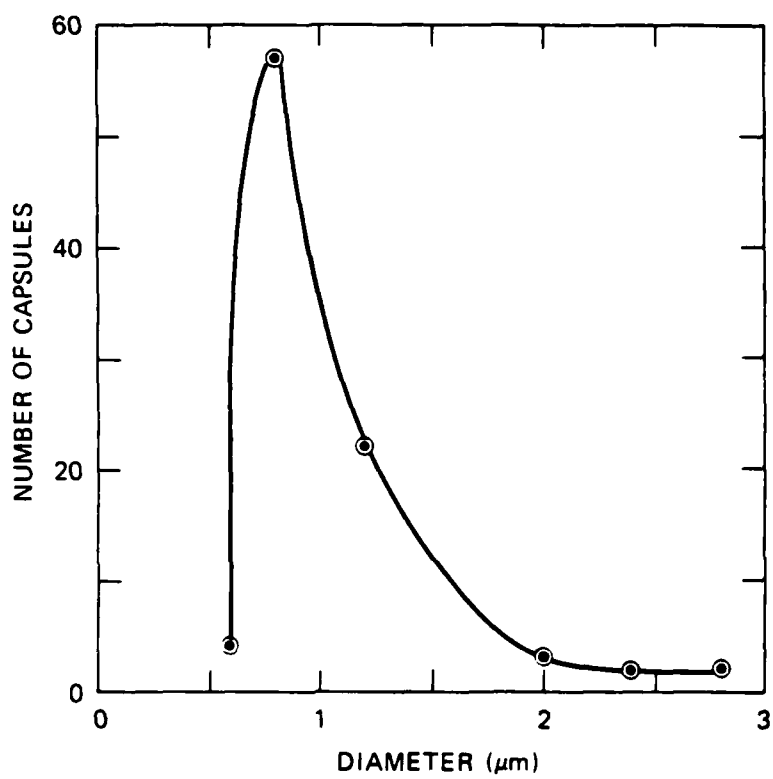
For determining the absorption spectrum of the microencapsulated Hb, 1.0-ml sample of the microcapsule suspension was diluted with 10 ml 0.9% NaCl. The absorption spectrum was taken immediately after dilution (Curve 1, Figure 2) by using an opaque cell in the reference beam. The spectrum was then determined after the sample had been stirred under argon in a bell jar for 30 min at room temperature while passing argon at the rate of 10 ml/min (Curve 2, Figure 2). Curves 3 and 4 show the changes in the spectrum of the microencapsulated Hb after passing oxygen at the rate of 10 ml/min for 15 and 30 min, respectively. No further change in the spectrum of Curve 4 was observed by passing oxygen for 14 min longer. The spectrum of Curve 2 was obtained again by passing argon for 30 min. This test shows that the Hb was not changed by the encapsulation.

The oxygen affinity of the microencapsulated Hb was measured by the biotometric method.\* The oxygen dissociation curve showed a  $P_{50}$  of 16 mm Hg, Figure 3. (In one test we obtained a  $P_{50}$  of 20 mm Hg; however, the system was malfunctioning.)

It was difficult to determine the actual concentration of Hb in the capsules. However, we extracted samples of the microcapsules with methylene chloride after they were washed with water. Methylene chloride extracted the EC, and the residue was treated with ethanol to precipitate the Hb and GA. From the dry weights of these two fractions, the EC was 59% and Hb plus GA was 41%. Total GA in the capsules is approximately 1%.

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\*J. R. Neville, J. Appl. Physiol. 37, 967-971 (1974).



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FIGURE 1 PARTICLE SIZE DISTRIBUTION OF MICROENCAPSULATED SFHb S-11860-17

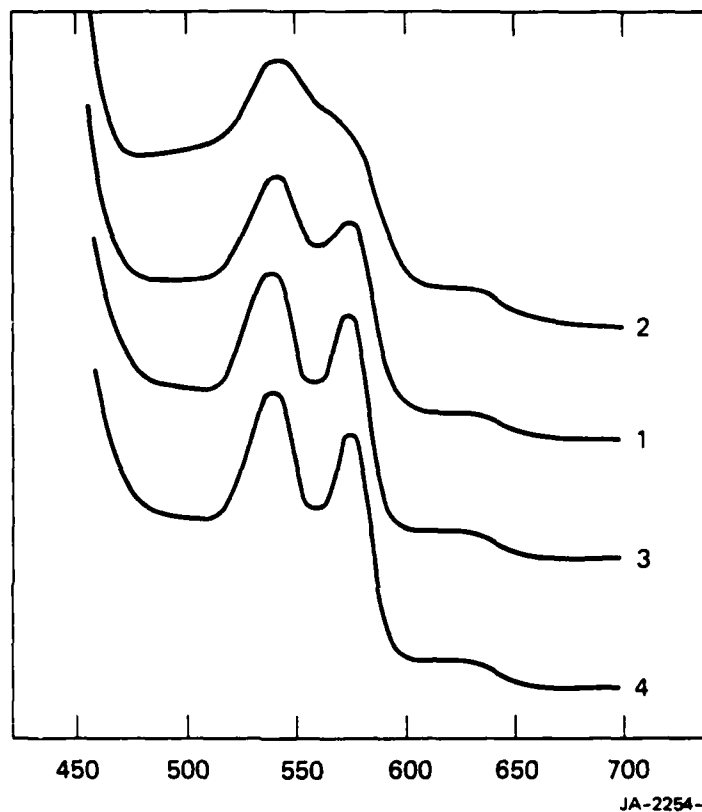
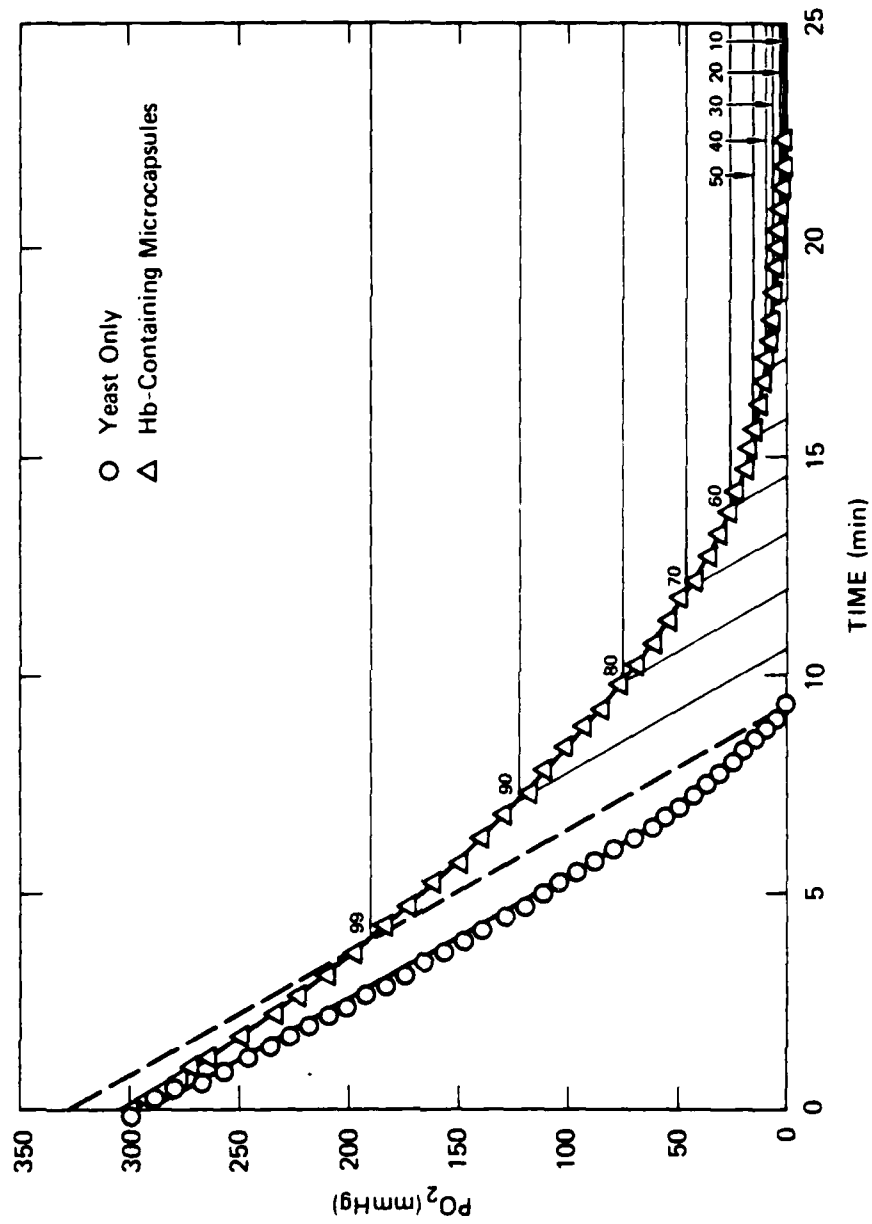


FIGURE 2 CHANGE OF ABSORPTION SPECTRUM BY OXYGENATION OF ENCAPSULATED Hb

1. Immediately after diluting the saline suspension
2. After passing argon for 30 min at the rate of 10 ml/min
3. After passing oxygen for 15 min at the rate of 10 ml/min
4. After passing oxygen for 30 min at the rate of 10 ml/min



JA-2254-3

FIGURE 3 OXYGEN DISSOCIATION OF Hb-CONTAINING MICROCAPSULES V. TIME

The concentration of the microcapsules in the suspension was determined by centrifuging a sample of the suspension and measuring the volume of the microcapsules and of the clear liquid layer. The microcapsule concentration was 16%. This value gives a Hb content of approximately 6%, which is higher than the spectrophotometric determinations indicated. Possibly the microcapsules were not completely separated from the saline when centrifugation was stopped.

## CONCLUSIONS AND RECOMMENDATIONS

Hb-containing microcapsules were prepared from all the encapsulating materials tested; however, only the EC-GA system yielded microcapsules of uniform size, good stability, and with Hb having properties comparable to those of the original Hb solution. Although the Hb content of the sample prepared was low, the system can be scaled up. We plan to prepare a larger batch of microcapsules with a higher Hb content for evaluation at LAIR.

The lecithin-protein-polysaccharides complexes are promising materials for the encapsulation of Hb; however, the encapsulation procedure is not as simple as that used for the EC-GA system, and a more detailed study of the process variables should be conducted. In addition, chemically modified proteins and polysaccharides, particularly ester or ethers, should be tested because the modified products are usually purer than the native ones, and their solubility characteristics can be modified to simplify the encapsulation procedure.

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